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Comparison between oleic acid and docosahexaenoic acid binding to interphotoreceptor retinoid-binding protein

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Abstract

Interphotoreceptor retinoid-binding protein (IRBP), which binds retinoids and fatty acids, is the major soluble protein in the interphotoreceptor matrix (IPM) but its role remains ambiguous. Using competitive fluorescence and tryptophan-quenching assays we found oleic acid and other *cis*-monounsaturated fatty acids bind much more strongly than does docosahexaenoic acid to bovine IRBP. IPM's fatty acid composition was determined: it was richer in oleic acid than either the retinal pigment epithelium or rod outer segments. This may imply oleic acid has a key role in the balance and transport of retinoids and fatty acids in the retina.

Keywords: Docosahexaenoic acid; Fatty acids; Fluorescence; Interphotoreceptor matrix; Interphotoreceptor retinoid binding protein

1. Introduction

All-*trans*-retinol is usually transported by carrier proteins which protect it from chemical degradation in both intracellular and extracellular aqueous compartments (Noy, 2000). A number of studies show that interphotoreceptor retinoid-binding protein (IRBP), which is confined to the interphotoreceptor matrix (subretinal space) of the retina, could be involved in the protection and diffusion of retinoids through the aqueous media (Pepperberg et al., 1993). Nevertheless, in vitro experiments have demonstrated that an intermembranous transfer of retinoids can occur independent of any retinoid-binding protein. In transgenic IRBP −/− mice, retinoid transfer still occurs, but the loss of photoreceptor cells observed showed that IRBP was still necessary for photoreceptor survival (Palczewski et al., 1999). Due to its high molecular weight and unusual shape for a diffusible protein carrier, IRBP could play a passive role in retinoid transport by preventing the degradation and potentially cytotoxic effects of free retinoids when large amounts are released into the interphotoreceptor matrix (IPM). The present work using fluorescence based ligand-binding assays relates to the interrelationships between binding of the two types of ligands, all-*trans*-retinol and long-chain FAs, to IRBP and the contribution of IRBP to the process of transfer of retinol and fatty acids in the aqueous media.

The association of all-*trans*-retinol with the hydrophobic retinoid-binding site of IRBP increases its fluorescence, which we measured using high sensitivity fluorimetry. We examined the effects of oleic acid and docosahexaenoic acid (DHA) on the interactions of IRBP with all-*trans*-retinol. The studies are focused on these two FAs, since the former was found to be a main constituent of IRBP-bound FA (29.4%), while its intracellular content in both ROS and RPE is relatively small (3.7% and 7.8%, respectively) (Bazan, Reddy, Redmond, Wiggert, & Chader, 1985), and the latter which occurs in large amounts in ROS, was reported to regulate the release of 11-*cis*-retinal from IRBP near the photoreceptor cells by binding to the protein (Chen, Saari, & Noy, 1993).

The displacement of all-*trans*-retinol by fatty acids, especially by oleic acid would only be physiologically relevant if these are present in the IPM. Therefore we quantified the fatty acids present in bovine IPM using gas chromatography (GC) and determined the amount of oleic acid present as phospholipids, neutral lipids and free fatty acids.

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2. Materials

IRBP. IPM was prepared from fresh bovine eyecups and excised retinas (<1 h after slaughter) which were incubated on the ice with PBS-P-A (phosphate-buffered saline, AEBSF, azide, pH 7.4) for 15 min and then clarified by centrifugation (Adler, Chader, & Wiggert, 1990). SDS gel electrophoresis of the IPM (supernatant) showed the ROS contamination, as estimated by intensity of the 35–40 kDa band, was under 10% (Al-Mahdawi et al., 1990). IRBP was purified from the IPM by Concanavalin A Sepharose affinity chromatography followed by a DEAE Sephacel column. The product was called 'native' IRBP since it was not treated further. SDS–polyacrylamide gel electrophoresis showed a single protein band at 140 kDa.

Fluorescence measurements. Fluorescence emission spectra were recorded at 20 °C with a SPEX FluorMax spectrofluorimeter (Spex Industries, Edison, NJ), using 2 ml samples in a silica cuvette. Fluorescence data were corrected for dilution where necessary, and fitted by standard nonlinear regression techniques (using Microcal ORIGIN software) to a single noncompetitive binding model, where appropriate, to give estimates of the dissociation constant (K_d) and the number of binding sites. Fluorescence monitors primarily the first, or hydrophobic, retinol binding site in IRBP (Noy, 2000).

Ligands. The following ligands, used for the binding studies, were all obtained from Sigma: all-*trans*-retinol, oleic acid, DHA, palmitoleic acid, *cis*-10-heptadecenoic acid, *cis*-10-nonadecenoic acid and *cis*-11-eicosenoic acid. The fatty acids were stored as stock solutions of approximately 10 mM in ethanol, in the dark at −20 °C, and freshly diluted in phosphate-buffer saline (PBS; 171 mM NaCl, 3.35 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) before use in the fluorescence experiments. Free retinol is poorly soluble and unstable in an aqueous environment, so was dissolved and diluted in ethanol immediately before use. The concentration of retinol was estimated by absorbance of a solution in ethanol at 325 nm, with an E_{325} of 52,480 $M^{-1}\,cm^{-1}$ (Szuts & Harosi, 1991).

Fluorometric titrations of IRBP with retinol. Binding of all-*trans*-retinol to protein was tested by addition of a 35 μM solution in ethanol in typically 5-μl aliquots directly to a cuvette containing 2 ml of 0.26 μM protein in PBS and mixing immediately. The final ethanol concentration was usually below 1%. The K_d was estimated with correction for the fluorescence of free retinol added to a cuvette containing only PBS. The interactions with the binding site were followed by monitoring the enhancement of the fluorescence of the retinol upon binding at this site (excitation, 350 nm; emission, 475 nm).

Competitive titrations of fatty acids with IRBP. These experiments were performed using reverse titration and quenching of tryptophan in IRBP. The reverse titrations were carried out by monitoring displacement of retinol from IRBP caused by the addition of increasing amounts of various fatty acids. Ligand binding was monitored by following the fluorescence of retinol (excitation, 350 nm; emission, 475 nm). Tryptophan quenching was performed by following the intrinsic fluorescence of protein by adding fatty acids directly in the cuvette containing protein dissolved in PBS (excitation, 280 nm; emission 330 nm). The final ethanol concentration did not exceed 1% at any time.

Determination of fatty acids in bovine IPM. The total lipids contained within the IPM were extracted using the procedure developed by Folch, Lees, and Stanley (1957). The chemicals were all obtained from Sigma aside from the standard lipid mix, PUFA-2, which was obtained from Supelco UK, Poole, Dorset. Solid phase extraction was performed to fractionate the total lipid extract into phospholipids, neutral lipids and free fatty acids by separation on Bond Elut NH_2 aminopropyl bonded solid phase columns (Burke Analytical, Alva, Scotland) (Kaluzny, Duncan, Merritt, & Epps, 1985). The lipid samples (total lipid and lipid fractions) were derivatised by reaction with 14% BF_3 in methanol and the fatty acid methyl esters were analysed by GC using the following equipment: a Hewlett Packard Series II gas chromatograph equipped with a flame ionisation detector and a Hewlett Packard HP 3395 integrator. Separation was achieved by the Supelco SP™ 2330 fused silica capillary column (0.2 μm, 30 × 0.32 mm). The chromatographic conditions were as follows: starting temperature 50 °C, increasing immediately at 30 °C/min to 155 °C where the temperature is held for 10 min. The temperature then increased at 10 °C/min to 220 °C where it was held for 5 min.

3. Results

3.1. Retinol binding to IRBP

The retinol binding activity of IRBP was investigated using the changes in retinoid fluorescence that occur upon interaction with the first retinoid-binding site of the protein (excitation, 330 nm; emission, 475 nm). The fluorescence emission of all-*trans*-retinol was minimal in buffer alone, but was dramatically enhanced when added to a solution of IRBP, as it moved from an aqueous environment to a more confining position within the binding site (Chen et al., 1993). The apparent dissociation constant of the retinol/IRBP complex was estimated by fluorescence titration in which increasing quantities of retinol were added to an IRBP solution in the cuvette. Data were fitted by standard non-linear regression analysis (using ORIGIN software) to a single non-competitive binding model to give estimates of the dis-

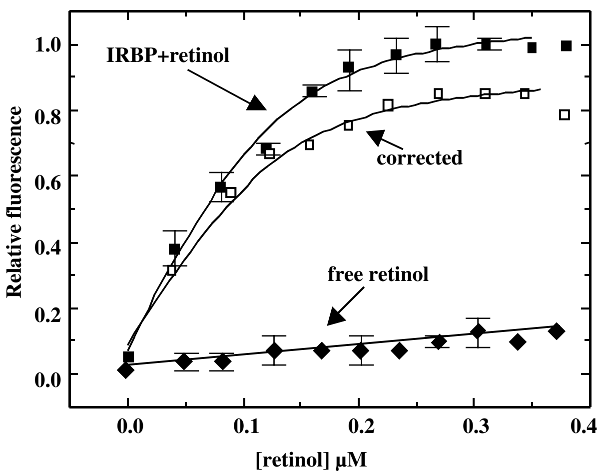

Fig. 1. Titration of IRBP with all-*trans*-retinol. Titrations were carried out in the absence (diamonds) or in the presence (closed boxes) of 0.26 μM IRBP, at $\lambda_{ex} = 350$ nm; $\lambda_{em} = 475$ nm. Open boxes show the corrected IRBP titration curve. Means and standard deviations ($n = 3$) are shown; where error bars are not shown, standard deviations are less than 0.01 units.

sociation constant (K_d) and the number of binding sites (n). The relevant equation is given below

$$F = F_1 + (F_2 - F_1) \cdot [(nC_o + C_L + K_d) - \{(nC_o + C_L + K_d)^2 - 4nC_oC_L\}^{1/2}]/2nC_o$$

In this equation, F is the fluorescence measured at a given ligand concentration C_L, F_1 is the fluorescence intensity in the absence of ligand and F_2 is the fluorescence intensity for the fully-bound state. C_o is the concentration of the protein (IRBP), which is fed into the computer programme in the same units as for the concentration of ligand. Note that the K_d's obtained are only apparent and thus approximate values.

Fig. 1 shows the dissociation curve, corrected for free retinol, consistent with binding at binding sites which have a K_d of 0.02 ± 0.02 μM and an n, the number of binding sites, of 0.48. The value of the K_d appears to be slightly lower than in previous studies (0.11 ± 0.02 μM) (Chen et al., 1993). The number of binding sites was less than one: some of the protein may have become inactive during preparation and storage.

3.2. Competitive titrations of fatty acids with IRBP

Binding of all-*trans*-retinol to native IRBP can be affected by the addition of fatty acids. It was previously reported by Chen et al. (1993) that the polyunsaturated fatty acid DHA was very effective in displacing retinol from IRBP. We performed reverse titrations to measure the extent of displacement of retinol by DHA and oleic acid from the binding site of native IRBP (Fig. 2). We found that the apparent dissociation constant obtained by measuring the dissociation of the IRBP–retinol complex by the addition of DHA ($K_{d(DHA)} = 2.56 \pm 0.07$ μM) was larger than the dissociation constant obtained for oleic acid ($K_{d(\text{oleic acid})} = 0.06 \pm 0.01$ μM), that is the DHA binding was weaker.

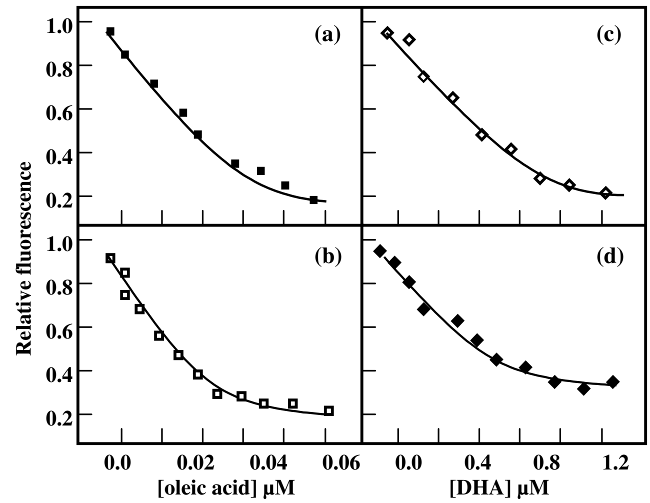

Fig. 2. Titration curves for binding of oleic acid and DHA to IRBP, obtained using reverse titration and intrinsic fluorescence quenching. (a) and (c) IRBP (0.26 μM) was incubated with all-*trans*-retinol (0.5 μM) and displaced by 0.0–0.06 μM oleic acid (a, closed boxes) or 0.0–1.2 μM DHA (c, open diamonds). Fluorescence was measured at $\lambda_{ex} = 350$ nm, $\lambda_{em} = 475$ nm; (b) and (d) quenching of tryptophan fluorescence in 0.26 μM IRBP by 0.0–0.06 μM oleic acid (b, open boxes) or 0.0–1.2 μM DHA (d, closed diamonds), using $\lambda_{ex} = 280$ nm, $\lambda_{em} = 330$ nm. Solid lines show the best fitting curves. Note that the range of oleic acid concentrations in a,b is 0.0–0.06 μM while that of DHA in (c) and (d) is 0.0–1.2 μM, that is, about 20 times more DHA than oleic acid is required to produce about the same effect.

Tryptophan fluorescence quenching was used to investigate the affinity of oleic acid for IRBP in the absence of retinol (using $\lambda_{ex} = 280$ nm; $\lambda_{em} = 330$ nm). It is known that all-*trans*-retinol could bind IRBP in two retinoid binding sites leading to quenching of the fluorescence of the tryptophan or tyrosine residue(s) which are located near both of the sites (Fedorovich, Semenova, Grant, Converse, & Ostrovsky, 2000). We found that the intrinsic fluorescence of IRBP was also decreased upon binding of oleic acid; this binding was characterised by an apparent dissociation constant of 0.04 ± 0.02 μM. The comparable value of the dissociation constant for DHA binding, also measured by quenching of intrinsic fluorescence, was found to be 1.1 ± 0.05 μM. Thus, again, DHA appears to have a lower affinity than oleic acid for IRBP.

3.3. Competitive titrations for the range of mono-unsaturated fatty acids

The finding that oleic acid (C 18:1) has a greater effect on retinol binding to IRBP than DHA (C 22:6) could be explained by the presence of a different number of unsaturated bonds which might be important for binding

Table 1
Apparent dissociation constants measured by either reverse titration[a] or by quenching of tryptophan (Trp) in IRBP ($n = 3$, ±SD)

Fatty acid	Reverse titration of IRBP[a] K_d (μM)	IRBP Trp quenching K_d (μM)
Palmitoleic acid (C 16:1)	0.038 ± 0.004	0.012 ± 0.004
Cis-10-heptadecenoic acid (C 17:1)	0.013 ± 0.003	0.018 ± 0.001
Oleic acid (C 18:1)	0.064 ± 0.012	0.036 ± 0.021
Cis-10-nonadecenoic acid (C 19:1)	0.031 ± 0.081	0.054 ± 0.014
Cis-10-eicosenoic acid (C 20:1)	0.089 ± 0.017	0.018 ± 0.010

[a] Retinol: IRBP = 2:1, displacement of all-*trans*-retinol by FAs monitored by fluorescence at 475 nm (ex. 350 nm).

Table 2
Results of total fatty acid analysis of bovine IPM

Fatty acid	Bovine IPM	
	I	II
Palmitic (16:0)	33.92	35.65
Palmitoleic (16:1)	3.30	3.51
Stearic (18:0)	18.08	18.16
Oleic (18:1)	15.56	15.74
Linoleic (18:2)	6.97	7.13
Linolenic (18:3)	0.62	0.28
Arachidonic (20:4)	10.58	10.08
DHA (22:6)	10.97	9.45
Total	100.00	100.00

Values are given as mol%. Results of two independent determinations are shown.

of fatty acids to IRBP at the site studied. If oleic acid, which possesses only one *cis*-unsaturated bond in its chain, has the largest effect, then the site may have structural requirements for binding mono-unsaturated fatty acids. To investigate the structural requirements of the site, we evaluated the competitive binding of a range of *cis*-unsaturated fatty acids with retinol. The IRBP-retinol complex was titrated in the presence of (C 16:1) *cis*-9-hexadecenoic acid (palmitoleic acid), (C 17:1) *cis*-10-heptadecenoic acid, (C 18:1) *cis*-9-octadecenoic (oleic acid), (C 19:1) *cis*-10-nonadecenoic acid and (C 20:1) *cis*-10-eicosenoic acid. The fatty acids probed were all *cis*-configuration and possessed only one unsaturated double bond. In parallel experiments the intrinsic fluorescence of IRBP was monitored after titrating the protein with increasing amount of fatty acids. Apparent dissociation constants for all the monounsaturated fatty acids studied (Table 1) were found to be similar, suggesting that the presence of the unsaturated bond is important for binding of fatty acids to IRBP and displacement of retinol from its binding site.

3.4. Determination of fatty acids in bovine IPM

The fatty acids extracted from bovine IPM were analysed using gas chromatography. The amount of oleic acid and quantities of each fatty acid identified in bovine IPM were calculated as molar percentages (Table 2). Our results are in agreement with previously obtained data for the frog IPM (Anderson, R.E., personal communication).

The unsaturated fatty acids 16:1, 18:1, 18:2, 18:3, 20:4 and 22:6 make up about 48% of the total bovine IPM fatty acid content, while saturated fatty acids (16:0 and 18:0) comprise about 52%. The principal saturated fatty acid is palmitic acid (16:0), which makes up about one-third of the total fatty acids. Arachidonic acid and DHA were present in the IPM in approximately equal amounts. The fatty acid composition of bovine IPM is similar in some respects to the composition of fatty acids endogenously bound to bovine IRBP, previously reported by Chen, Houghton, Brenna, and Noy (1996). They found that the major fatty acids associated with bovine IRBP were palmitic (26.6%), stearic (13.5%) and oleic (29.4%) acids. The polyunsaturated FAs bound to IRBP were 7.7% of the total (arachidonic acid) and 8.6% (DHA). Similar results were reported by Bazan et al. (1985) for monkey IRBP.

From the total lipid extract, the amount of oleic acid in the IPM of one bovine eye was calculated to be on average 34.8 μg. To compare, the solid phase extraction method revealed that the amount of oleic acid present as free fatty acid was 13.5 μg, the amount of oleic acid in the phospholipid fraction was 12.6 μg and in the neutral lipid fraction was 3.9 μg. Therefore, the total amount of oleic acid in these various fractions was found to be 30.0 μg, which is close to the results obtained from the total lipid extract. This relatively large amount of oleic acid in IPM compared to DHA, and compared to oleic acid in ROS and RPE (Chen et al., 1996), is relevant to the discussion of fatty acid binding which follows.

4. Discussion

IRBP is the major soluble protein of the interphotoreceptor matrix and could be isolated using two types of chromatographic techniques in succession: Concanavalin A Sepharose column chromatography and DEAE Sephacel column chromatography. The isolated protein is reported to display the same properties with respect to molecular weight and retinol binding as it does when present in unfractionated interphotoreceptor matrix, and can be used for ligand-binding studies (Chader, 1989).

Fluorescence-based studies show that the protection and solubilisation of vitamin A in an aqueous environment might be accomplished by binding to IRBP but this binding is affected by a variety of ligands. The fluorescence studies conducted previously (Chen et al., 1993, 1996) indicated that long chain fatty acids inhibit binding of retinol to its stronger site on IRBP but quite a significant excess of fatty acid over protein, 10:1, was required to displace retinol from IRBP. DHA (C 22:6 *n*—3) was found to have the largest effect with a K_d of 0.78 μM; other fatty acids studied showed even smaller effects: oleic acid $K_d = 1.45$ μM, retinoic acid $K_d = 1.53$ μM and arachidonic acid $K_d = 1.64$ μM. The research from Noy's group was mainly concerned with DHA binding to IRBP; this binding was postulated to regulate the release of 11-*cis*-retinal from IRBP near the photoreceptor cells by binding to the protein. Using high sensitivity fluorimetry we found that oleic acid has higher affinity for IRBP than does DHA. We have focused mainly on oleic acid, since it comprises a high proportion of the FAs bound to IRBP (Bazan et al., 1985; Chen et al., 1996). The oleate content of lipids in ROS and RPE is relatively small (3.7% and 7.8%, respectively), whereas it represents a large fraction of IRBP-bound FAs, 29.4%. Using gas chromatography we found that oleic acid is the most abundant of the unsaturates in the IPM (~16% of the total fatty acids)—accounting for about one-third of the unsaturated fatty acids—and thus it may play an important role, directly or indirectly, in the retina.

Several fatty acids were capable of displacing vitamin A from IRBP; however, using fluorescence-based studies we found a remarkably high selectivity for oleic acid which was comparable in magnitude to the affinity of IRBP to its natural ligand all-*trans*-retinol. The question of whether oleic acid competes with retinol in binding to the same site remains unresolved. It is possible that the fatty acid binds to IRBP in a different location, and the resulting changes in the protein are followed by displacement of retinol. IRBP shows various degrees of relative affinity for fatty acids depending on the binding sites (Bazan et al., 1985; Chen et al., 1996). These could be solely fatty acid, or retinoid-fatty acid binding sites. The apparent low K_d for oleic acid, which possesses only one unsaturated bond, suggests that the presence of the single unsaturated bond is important for the binding of fatty acids to IRBP at the site studied. All the monounsaturated *cis*-fatty acids studied had similar apparent dissociation constants.

The significance of the selective affinity of oleic acid for IRBP is not yet clear. Although its role is not completely understood, IRBP has been thought to protect 11-*cis*-retinal and all-*trans*-retinol while facilitating their transport between the photoreceptors and retinal pigment epithelium (Chader, 1989). Experiments with IRBP −/− mice show rhodopsin regeneration still occurs normally, so IRBP is presumably not necessary for retinoid transport, though it still is important for photoreceptor survival (Palczewski et al., 1999). It may be that IRBP's role as a fatty acid transporter, not a retinoid transporter, is what is crucial to this survival. In this regard, it is interesting to postulate that oleic acid, which is present in sizeable amounts in the IPM and binds strongly to IRBP, displacing retinol, may play a key role in controlling IRBP's various functions in the retina.

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